PATENT
VAN67 P329

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	:	1635
Applicant	:	George F. Vande Woude et al.
Appln. No.	:	10/563,616
Filing Date	:	August 9, 2006
Examiner	:	Goddard, Laura B.
Conf. No.	:	1900
For	:	INHIBITION OF TUMOR ANGIOGENESIS BY COMBINATION OF THROMBOSPONDIN-1 AND INHIBITORS OF VASCULAR ENDOTHELIAL GROWTH FACTOR

DECLARATION UNDER 37 C.F.R. § 1.131

We, the undersigned, do hereby declare as follows:

1. We are the co-inventors of the claims of the above-identified patent application.

2. The invention as defined in claims 1, 5, 7-13, 16-18, 20-22, 26, 28-34, 37, 38, and 40-46 was conceived prior to March 8, 2002, and we were was reasonably diligent in reducing the invention to practice from prior to March 8, 2002, until the filing of our priority application on July 7, 2003.

3. Evidence of our conception and reasonable diligence in reducing to practice the invention as defined in claims 1, 5, 7-13, 16-18, 20-22, 26, 28-34, 37, 38, and 40-46 is provided in the form of experimental data from the laboratory notebooks of Yu-Wen Zhang, one of the named inventors (attached hereto as Exhibit A1-A17). More specifically, these laboratory notebooks show our development of a composition and method for inhibiting tumor angiogenesis comprising TSP-1 and a VEGF inhibitor, including:

a) Constructing expression vector pcDNA3/hygro-TSP-1 (Exhibit A1);

b) Transfecting SK-LMS-1 cells (SK/HGF cells) with pcDNA3/hygro-TSP-1 to obtain stable ectopic expression of TSP-1 (Exhibit A2);

c) Amplifying and purifying VEGF DNA fragment for a probe to confirm VEGF upregulation by HGF/SF (Exhibit A3);

d) Conducting RT-PCR to confirm expression of TSP-1 in stably expressed cell line (Exhibit A4);

e) Preparing RNA from cells with or without stable expression of TSP-1 (Exhibit A5);

f) Confirming HGF/SF expression in SK/HGF-TSP-1 cells (Exhibit A6);

g) Northern blot confirmation of TSP-1 expression in the SK/HGF cells stable transfected with TSP-1 (Exhibit A7);

g) In vivo mouse experiments to determine the effects of TSP-1 on tumor growth (Exhibit A8);

h) Preparing RNA from cells treated with inhibitors (Exhibit A9);

i) Conducting colony formation assays (Exhibit A10);

j) Northern blot analysis of TSP-1 and VEGF expression (Exhibits A11 and A12);

k) Northern blot analysis of TSP-1 expression in SK-LMS-1 cells inhibited by MAP kinase inhibitors (Exhibit A13);

l) Northern blot analysis of VEGF expression in SK-LMS-1 cells inhibited by MAP kinase inhibitors (Exhibit A14);

m) Preparation of protein lysate from SK-LMS-1 cells treated with MAP kinase inhibitors (Exhibit A15);

n) Conducting IHC staining of CD31 in tumors derived from mouse study for determining the effects of TSP-1 on tumor angiogenesis (Exhibit A16); and

o) Demonstrating the regulation of VEGF and TSP-1 by HGF/SF in MDA-MB-231 cells (Exhibit A17)

4. The documents attached as Exhibits A1-A17 were prepared contemporaneously with our conception and reasonable diligence in reducing the invention to practice.

5. In the first part of June, 2003, our patent attorney was contacted to begin preparation of related provisional application No. 60/484,676, which was filed in the U.S. Patent and Trademark Office on July 7, 2003.

6. The acts referred to in the preceding paragraphs occurred in the United States.

7. The undersigned hereby declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Sections 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

7-21-10
Date

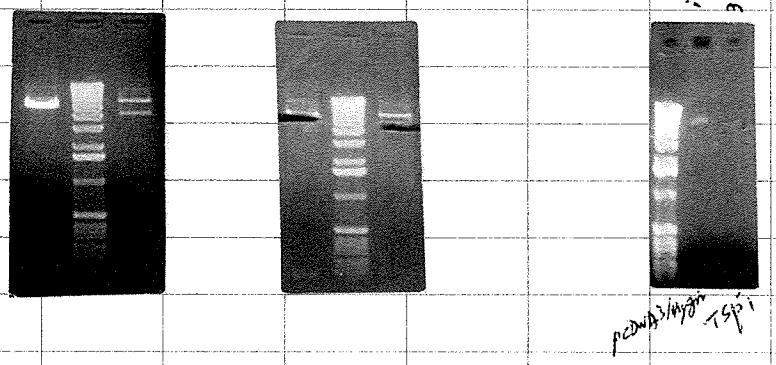
George F. Vande Woude

7/21/2010
Date

Yu-Wen Zhang

2/19/02 Construction for pcDNA3.1/Hygro - TSP1.

pcDNA3.1/Hygro (+)	6 μl	pcDNA1 - TSP1	15 μl
10X NE buffer 2	3	10X NE buffer 2	3
10X BSA	3	10X BSA	3
DW	15	DW	6
Hind III	1.5	Hind III	1.5
Xba I	1.5	Xba I	1.5
	30 μl		30 μl



gel purified.

ligation:

pcDNA3/Hygro (HindIII/XbaI)	0.5 μl
TSP1 fragment (")	14.5 μl
5 x ligase Reaction buffer	4 μl
DW	0
T4 DNA ligase	1 μl
	20 μl

RT, 1 hour.

transformation: 2 μl reaction mixture → 1 vial one shot (TOP1
ice 30 min → pulse at 42°C for 30 second.

Hygromycin [illegible]

2/26/02 Transfection of sk/HGF cells with pcDNA3-Hygro-TSP1

① pcDNA3-Hygro vector (1.7 kb)
③ pcDNA3-Hygro-TSP1 (11.7 kb)
+ Fugene 6 ml

transfection, 12:40 pm, 2/26/02

2/28/02 split cells for Hygromycin B selection. 400 ug/ml, 600, 800, 1000

3/4/02 Change medium with Hygro

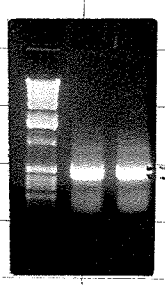
3/7/02 Change medium with Hygro

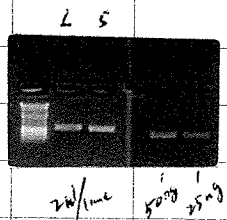
3/13/02 pick colonies for hygro-control &
hygro-TSP1

pool stock: sk/HGF-Hygro (800 ug/ml, 2wks) - 1 vial
sk/HGF-TSP1 (800 ug/ml, 2wks) - 1 vial

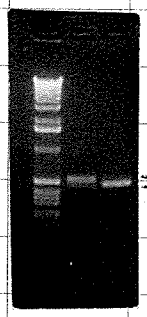
3/01/02 One step RT-PCR for VEGF probe

2 x Reaction mix	25 µl
Vegf-S (10 µM)	1 µl
Vegf-as (10 µM)	1 µl
SK/HUF total RNA (1 µg/µl)	1
DEPC-treated dw	21
RT/PLATINUM Taq mix	1
	50 µl





2µl/lane 50ng 25ng



use VEGF-S as probe

sequencing:

① VEGF-L ; Vegf-s primer
② VEGF-L ; Vegf-as primer
③ VEGF-S ; Vegf-s primer
④ VEGF-S ; Vegf-as primer
5. pcDNA3-Hygro-TSP1 ②, T7 primer
6. pcDNA3-Hygro-TSP1 ③, T7 primer

2µl on
1µl primer
(3.2 pm

3/15/02 Thawing sk/HGF-TSP clones (①, ⑤, ⑭, ㉖)
on growing ㊲.

sk/HGF-hygro clones (1, 3, 6)

3/15/02 RT-PCR to check the expression of TSP1

2 x Reaction mix	12.5
TSP-1 primer (20 μM)	0.5
TSP-2 primer (10 μM)	0.5
RNA sample (1 μg/μl)	0.5
DEPC-DW	10.5
RT/platinum Taq mix	0.5
	25 μl

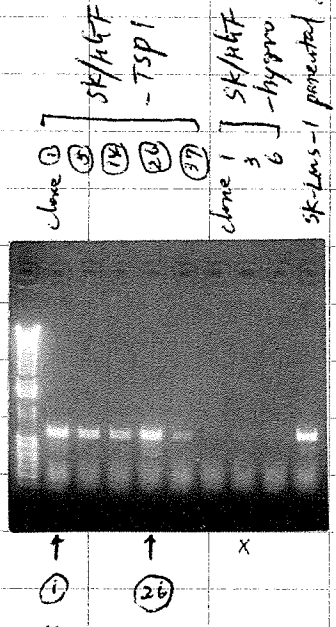
Method: #20. (21-22-23-24)

50°c, 30'] x 1 cycle
94°c, 2']

94°c, 15"]
55°c, 30"] x 30 cycles
72°c, 1']

72°c, 10' — 1 cycle.

4°c, ∞



8/19/02 Reisolate RNA for RT-PCR

?■ 2nd. 8/19/02

eppendorf BioPhotometer 6131 00094
08/19/2002 1/200 dilution V1.01

17:02 BLANK 0.000 A

17:03 #001 23.0 µg/mL RNA
SK/HGF. Hygro-1 4.6 µg/µl
0.217 A_{230}
0.575 A_{260}
1.36 260/280 0.422 A_{280}
2.65 260/230 0.001 A_{320}

17:05 #002 18.3 µg/mL RNA
SK/HGF. Hygro-3 3.66 µg/µl
0.172 A_{230}
0.458 A_{260}
1.36 260/280 0.337 A_{280}
2.67 260/230 0.002 A_{320}

17:06 #003 13.7 µg/mL RNA
SK/HGF. Hygro-6 2.74 µg/µl
0.137 A_{230}
0.344 A_{260}
1.35 260/280 0.254 A_{280}
2.51 260/230 0.003 A_{320}

17:07 #004 11.8 µg/mL RNA
SK/HGF. TSP-(5) 2.36 µg/µl
0.114 A_{230}
0.296 A_{260}
1.35 260/280 0.219 A_{280}
2.61 260/230 0.002 A_{320}

17:09 #005 15.8 µg/mL RNA
SK/HGF. TSP-(14) 3.16 µg/µl
0.153 A_{230}
0.394 A_{260}
1.37 260/280 0.288 A_{280}
2.58 260/230 0.000 A_{320}

17:11 #006 20.2 µg/mL RNA
SK/HGF. TSP-(26) 4.04 µg/µl
0.195 A_{230}
0.504 A_{260}
1.37 260/280 0.369 A_{280}
2.59 260/230 0.001 A_{320}

EXHIBIT A5

10/8/02 RT-PCR for detecting HGF in sk/HGF-TSP1 cells.

		x 10
2x Reaction mix	12.5	125
hHGF-X (10μM)	0.5	5
hHGF-Y (10μM)	0.5	5
RNA sample (1μg/μl)	1	
DW (DEPC)	10	100
RT/PLATINUM Taq mix	0.5	5
	25 μl	240 μl → 24 μl/samp + 1 μl RNA

Method. #20 (21-22-23-24)

50°C, 30'] x 1 cycle.
94°C, 2']

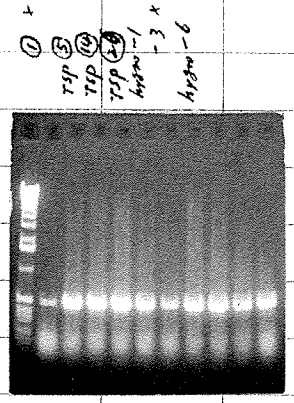
94°C, 15"]
55°C, 30"] x 30 cycles
72°C, 1']

72°C, 10' — 1 cycle

4°C, ∞

Samples:

1. sk/HGF-TSP — ①
2. " — ⑤ ✓
3. " — ⑭ ✓
4. " — ㉖ ✓
5. sk/HGF-hygro — 1 ✓
6. " — 3
7. " — 6 ✓
8. sk/HGF parental
9. sk-Lms-1 parental
10. sk-Lms-1 parental



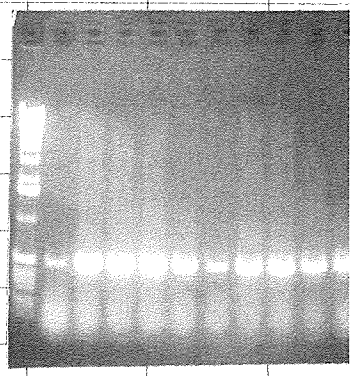


EXHIBIT A6

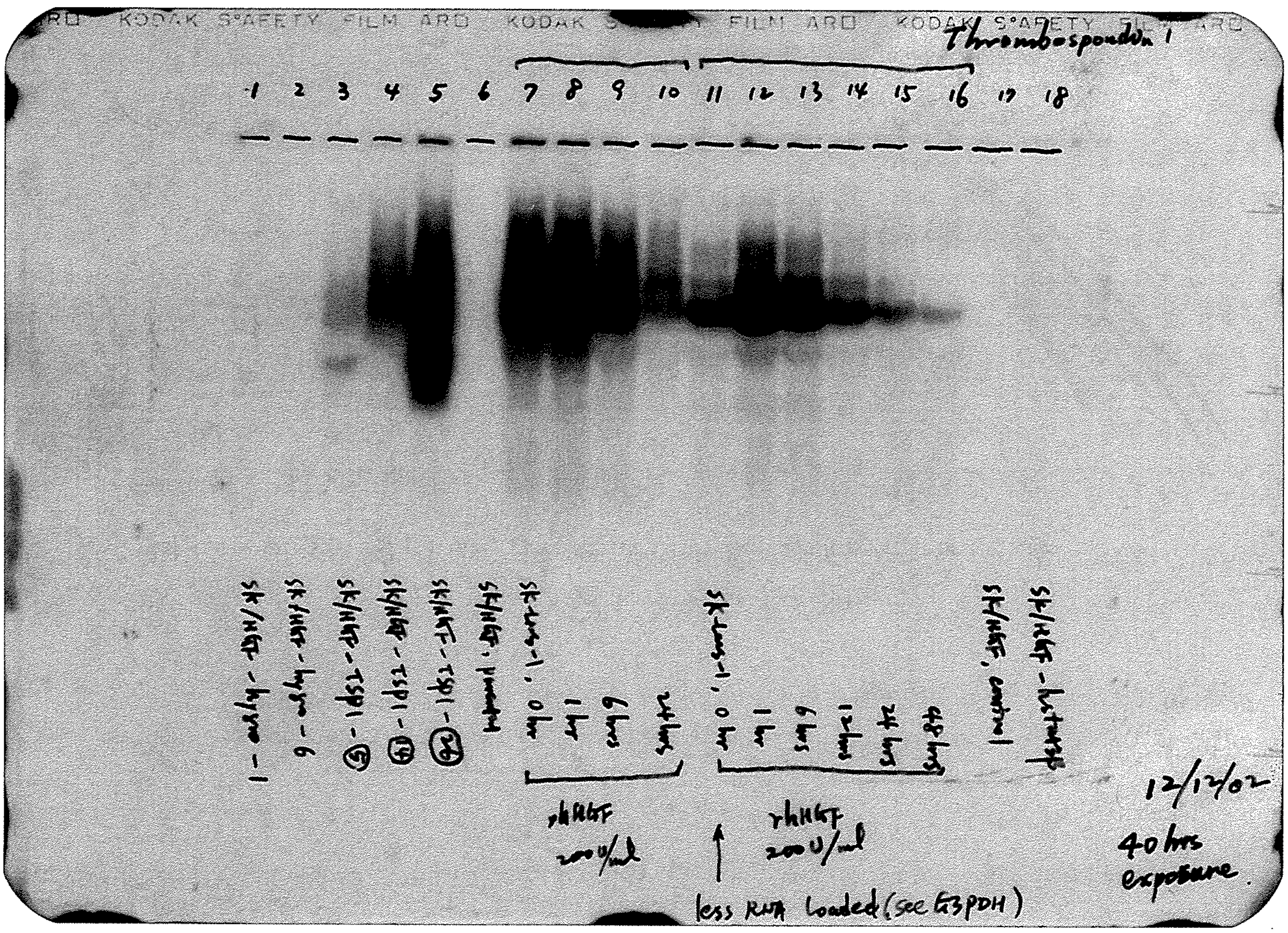

KODAK SAFETY FILM
Thrombospondin 1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
rhHGF 200U/ml
rhHGF 200U/ml
less RNA loaded (see G3PDH)
12/12/02
40 hrs exposure.

EXHIBIT A7

Injection of Nude Mice **1/28/2003**

5 mice for one group;
1 x 10^6 cells for one mouse;
Prepare cells in the concentration of 1 x 10^6 cell / 100 μl in DMEM without serum.
Subcutaneous injection

Samples:

1. SK-LMS-1
2. SK/HGF (SK-LMS-1 autocrine with HGF)
3. SK/HGF-TSP1 (clone 26)
4. C2C12
5. C2C12-hMet (clone 8)
6. C2C12-hMet+hHGF (clone 1)

1. Growing cells in a large frask.
2. Trypsinized cells and pelleted by centrifuge.
3. Resuspending cells in DMEM to the concentration of 1x 10^6 cells/ 100 μl.
4. Injecting nude mice (100 μl/mice) by Subcu.
5. Measuring tumor sizes every 3 days.
6. Observing tumors 2-3 weeks after injection.

2/3/03 Treat Sk-Lms-1 cells with MAPK and PI3 inhibitors in the presence of HGF (200 U/ml) in ~~20ml~~ DMEM + 10% FBS.

		final conc.	Amount /20ml
1.	DMSO		80 µl
2.	PD98059 (20mM stock)	80 µM	80 µl
3.	U0126 (10 mM stock)	40 µM	80 µl
4.	LY294002 (10mM stock)	40 µM	80 µl

2:00pm After treating cells with inhibitors for 1 hour, add HGF. for HGF (100 U/µl stock), add 40µl to each 20ml medium. Incubate for 24 hrs.

2/4/03 2:00pm Isolate total RNA

eppendorf BioPhotometer 6131 30094
02/04/2003 1/200 dilution v1.01

17:31 BLANK Sk-Lms-1 (+) rHGF 0.000 A 24 hours

17:33 #001 35.8 µg/mL RNA
(+) DMSO 7.16 µg/µl
0.343 A230
0.895 A260
1.83 260/280 0.642 A280
2.61 260/230 0.005 A320

17:33 #002 36.6 µg/mL RNA
(+) PD98059 7.32 µg/µl
0.354 A230
0.916 A260
1.40 260/280 0.653 A280
2.59 260/230 0.000 A320

17:35 #003 42.6 µg/mL RNA
(+) U0126 8.52 µg/µl
0.416 A230
1.064 A260
1.41 260/280 0.757 A280
2.56 260/230 0.003 A320

17:36 #004 27.5 µg/mL RNA
(+) LY294002 5.5 µg/µl
0.265 A230
0.689 A260
1.38 260/280 0.499 A280
2.60 260/230 0.001 A320

EXHIBIT A9

3/5/03 Colony formation assay in soft agar. (Triplicate)

1. SK-Lms-1 Control cells.
2. SK/HGF Control cells.
3. SK/HGF-TSP1 cl(26).

22-203

3/19/03 Count colony numbers under phase contrast microscope.
(size > 0.1 mm; 1000 cells from each plate were counted)

	set 1	set 2	set 3
SK-Lms-1 Control	8	12	19
SK/HGF control	512	465	538
SK/HGF-TSP1 cl(26)	474	525	557

3/18/03 Northern blot

		(μg/μl) Conc.	(10 μg) RNA	Dw up 5.5 μl
1.	sk/HGF-hypro-6	2.74	3.65	1.85
2.	sk/HGF-TSP1-(26)	4.04	2.48	3.02
3.	sk/HGF, parental	5.47	1.83	3.67
4.	sk-Lms-1 (no HGF), 0 hr	4.46	2.24	3.21
5.	sk-Lms-1 (rHGF), 1 hr	4.34	2.3	3.2
6.	sk-Lms-1 (rHGF). 6 hrs	4.6	2.17	3.3
7.	sk-Lms-1 (rHGF), 24 hrs	5.26	1.9	3.6
8.	sk-Lms-1, rHGF, 1 hr	10	1	4.5
9.	" , 6 hrs	10	1	4.5
10.	" , 12 hrs	10	1	4.5
11.	" , 24 hrs	10	1	4.5
12.	" , 48 hrs	10	1	4.5
13.	sk/HGF. control	5.47	1.83	3.6
14.	sk/HGF - hstat3β.	4.08	2.45	3.0
15.	sk-Lms-1 (no HGF), 0 hr	4.46	2.24	3.2
16.	sk-Lms-1, rHGF, 24 hrs	10	1	4.5
17.	sk-Lms-1 (rHGF, 24 hrs), DMSO	7.16	1.4	4.1
18.	" , PD98059	7.32	1.37	4.1
19.	" , U0126	8.52	1.17	4.3
20.	" , LY294002	5.5	1.82	3.6

Loading:

RNA in	5.5 μl
10XMOPS	1
37% formaldehyde	3.5
formamide	10

→ premix 14.5 μl/sample

3/21/03 Hybridization with human TSP1.

3/22/03 stripping membrane in boiled 500 ml of 0.1% SDS solution

↓

slowly cool down to RT (under 30°C)

↓

rinse with 2x SSC buffer.

↓

Rehybridization with human VEGF probe.

3/21/03 Hybridization with human TSP1.

3/24/03 stripping membrane in Boiled 500ml of 0.1% SDS solution

↓

slowly cool down to RT (under 30°C)

↓

rinse with 2xSSC buffer.

↓

Rehybridization with human VEGF probe.

1. Sk/NKF-hygro
2. Sk/NKF-TSP1
3. Sk/NKF- parental
4. Sk-Lms-1, no NKF
5. rNKF, 1 hr
6. rNKF, 6 hrs
7. rNKF, 24 hrs
8. rNKF, 1 hr
9. rNKF, 6 hrs
10. rNKF, 12 hrs
11. rNKF, 24 hrs
12. rNKF, 48 hrs
13. Sk/NKF, control
14. Sk/NKF- bacteria
15. Sk-Lms-1, no NKF
16. Sk-Lms-1, rNKF- 24 hrs
17. DMSO
18. PD98059
19. U0126
20. LY294002
Sk-Lms-1
rNKF, 24 hrs
TSP1
40 hrs exposure (i.e. Screen)

EXHIBIT A13

1. Sk/HGF -Hygro
2. Sk/HGF-Tsp1
3. Sk/HGF parental
4. Sk-Lung-1, no HGF
5. rHGF, 1 hr
6. rHGF, 6 hrs
7. rHGF, 24 hrs
8. rHGF, 1 hr
9. rHGF, 6 hrs
10. rHGF, 12 hrs
11. rHGF, 24 hrs
12. rHGF, 48 hrs
Sk-Lung-1
13. Sk/HGF, control
14. Sk/HGF - hStat3β
15. Sk-Lung-1, no HGF
16. Sk-Lung-1, rHGF 24 hrs
17. Dmso
18. PD98059
19. U0126
20. LY294002
rHGF, 24hrs
Sk-Lung-1
VEGF
48 hours
exposure
2/27/03

EXHIBIT A14

preliminary map of [illegible]

3/28/03 Sk-Lms-1 treated with inhibitor and/or HGF.

Sk-Lms-1 cells were cultured in DMEM + 0.1% BSA for O/N (Serum starvation).

Add inhibitors.

			HGF (200
1.	--		-
2.	--		+
3.	DMSO	20 µl/5 ml	+
4.	PD98059 (10 mM stock)	20 µl	+
5.	U0126 (10 mM stock)	20 µl	+
6.	LY294002 (10mM stock)	20 µl	+

↓

Incubate cells with inhibitor for 1 hour

↓

Add 200 U/ml of HGF to each plate.

↓

Incubate for 15 min.

↓

prepare cell extracts in RIPA buffer (with P.I.) (in 750 µl)

Washing cells three times with ice cold 1 x PBS

↓

Add 750 µl RIPA buffer to each 10 cm dish

↓

Harvest cell lysate using cell scraper and keep in 1.5 ml tube

↓

Rotate at 4°C for 15 min.

↓

Freeze in liquid nitrogen for 5 min.

↓

Thaw in ice water

↓

Centrifuge at 13500 rpm. 4°C for 15 min

↓

Collect supernatant and quantify the protein concentration

rat ABC staining system (Santa Cruz)

4/17/03 Immunohistochemistry staining with rat anti-mouse CD31 antibody

1). Formalin-fixed paraffin block section (5 micron). (secondary = anti-rat IgG)

2) Deparaffinize: 3 x Xylene 2 min each
2 x 100% ethanol
2 x 95% ethanol

3) Block: 1 hour in 1.5% normal serum in 1x PBS

4) primary antibody: rat anti-mouse CD31. 1:20 dilution in blocking buffer.
incubate at 4°C for O/N
Wash three changes of 1x PBS for 5 min each

5) Biotinylated secondary antibody: 30 min at RT
Wash three changes of 1x PBS for 5 min each.

6) AB enzyme reagent: 30 min at RT.
wash three changes of 1xPBS for 5 min each.

7) Peroxidase substract. add 1~3 drops of peroxidase substract to each section
incubate for 10 min or until desired stain intensity develop
wash section in DW for 5 min.

8) Hematoxylin counter staining: 10 seconds
Immediately wash several times in DW.

9) Dehydrate section (for paraffin-embedded tissue section).
2 x 95% ethanol for 10 seconds each.
2 x 100% ethanol for 10 seconds each.
3 x xylenes for 10 seconds each. Wipe off excessive

10) Immediately add 1~2 drops of permanent mounting medium and cover with a glass coverslip.

EXHIBIT A16

5/5/03 Northern blot.

	Conc. (ng/μl)	RNA (20 μg)	DW up to 5.5 ml
1. MDA 231 control-1	10.38	1.93	3.57
2. MDA 231 (+) HGF 24 hrs	11.12	1.8	3.7
3. MDA 231 (+) HGF 48 hrs	9.64	2.07	3.43
4. empty	—	—	—
5. MDA231 control-1	10.38	1.93	3.57
6. MDA 231 (+) HGF 24 hrs	11.12	1.8	3.7
7. MDA 231 (+) DMSO (+) HGF 24 hrs	10.96	1.82	3.68
8. MDA 231 (+) PD98059 (+) HGF 24 hrs	7.34	2.72	2.78
9. MDA 231 (+) U0126 (+) HGF 24 hrs	6.8	2.94	2.56
10. MDA 231 (+) LY294002 (+) HGF 24 hrs	7.28	2.75	2.75
11. empty	—	—	—
12. DBTRG control-1	7.64	2.62	2.88
13. DBTRG (+) HGF 24 hrs	7.66	2.61	2.89
14. DBTRG (+) HGF 48 hrs	10.02	2.0	3.5
15. —	—	—	—

5/6/03 Hybridization with human TSP1 probe (exposure: 5/8/03)

5/8/03 Hybridization with human VEGF probe after stripping the membrane (exposure: 5/10/03)

5/13/03 Hybridization with human GAPDH probe (exposure: 5/15/03)